

The cell cycle–apoptosis connection revisited in the adult brain

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Adult neurogenesis is studied *in vivo* using thymidine analogues such as bromodeoxyuridine (BrdU) to label DNA synthesis during the S phase of the cell cycle. However, BrdU may also label DNA synthesis events not directly related to cell proliferation, such as DNA repair and/or abortive reentry into the cell cycle, which can occur as part of an apoptotic process in postmitotic neurons. In this study, we used three well-characterized models of injury-induced neuronal apoptosis and the combined visualization of cell birth (BrdU labeling) and death (Tdt-mediated dUTP-biotin nick

end labeling) to investigate the specificity of BrdU incorporation in the adult mouse brain *in vivo*. We present evidence that BrdU is not significantly incorporated during DNA repair and that labeling is not detected in vulnerable or dying postmitotic neurons, even when a high dose of BrdU is directly infused into the brain. These findings have important implications for a controversy surrounding adult neurogenesis: the connection between cell cycle reactivation and apoptosis of terminally differentiated neurons.

Introduction

The adult mammalian brain contains neural stem/progenitor cells that proliferate and generate new neurons and glia, both *in vitro* and *in vivo* (Gage, 2000; Doetsch, 2003). Although neural stem cells reside in various areas of the nervous system (Taupin and Gage, 2002), adult neurogenesis has been unambiguously demonstrated *in vivo* in only two well-defined regions, the olfactory bulb and the hippocampus, in rodents and primates, including humans (Peterson, 2002).

The study of adult neurogenesis is limited by currently available techniques for identifying newly generated cells. *In vivo*, thymidine analogues such as tritiated thymidine or BrdU, which are incorporated into nuclear DNA during the S phase of the cell cycle, are often used to label proliferating cells (Rakic, 2002a). BrdU is very useful because it is revealed by immunohistochemistry, which can be combined with cell type-specific markers and confocal microscopy to identify the phenotypes of newly generated cells.

However, BrdU may also be incorporated during DNA synthesis not related to cell proliferation, such as normal DNA turnover or DNA repair (Nowakowski and Hayes, 2000; Cooper-Kuhn and Kuhn, 2002; Rakic, 2002a,b). In addition, a growing

number of studies show that terminally differentiated neurons can reenter the cell cycle and incorporate BrdU as part of an apoptotic process (Copani et al., 2001; Becker and Bonni, 2004; Greene et al., 2004; Herrup et al., 2004), which is apparently triggered by the DNA damage response (Kruman et al., 2004). For instance, key regulators of the cell cycle, such as cyclins and Cdks, are up-regulated in postmitotic neurons subjected to a variety of death-inducing stimuli *in vitro* or *in vivo*, as well as in Alzheimer's disease (AD; Greene et al., 2004), where vulnerable neurons could initiate abortive DNA synthesis without cell division before undergoing cell death (Yang et al., 2001). Preventing the induction of cyclin D1 or Cdk4 protects against excitotoxin-induced neuronal death *in vivo* in the adult rat, demonstrating the direct involvement of cell cycle regulators in the death of terminally differentiated neurons (Ino and Chiba, 2001).

Such findings raise the question of whether BrdU could be incorporated *in vivo* into dying postmitotic neurons and ultimately lead to false positive staining when studying neurogenesis (Rakic, 2002a). Clarification is needed because these uncertainties undermine the discovery of new areas in the adult brain, including the septum, hypothalamus, striatum (Pencea et al., 2001), amygdala (Bernier et al., 2002), and dorsal vagal complex (Bauer et al., 2005), where ongoing neurogenesis may occur at low levels and requires a higher dose of BrdU and/or repeated injections to be visualized. More controversial still are the substantia nigra (Kay and Blum, 2000; Lie

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Abbreviations used in this paper: AD, Alzheimer's disease; CA, cornu ammonis; casp-3, activated caspase 3; CldU, chlorodeoxyuridine; IdU, iododeoxyuridine; KA, kainic acid; OBX, olfactory bulbectomy; OE, olfactory epithelium; SGZ, subgranular zone; SVZ, subventricular zone.

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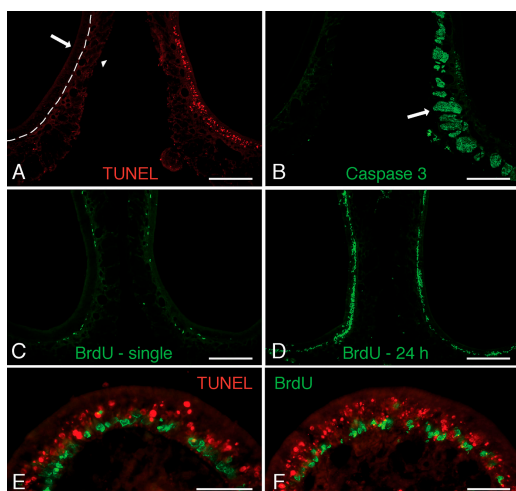


Figure 1. Validation of the BrdU/TUNEL double staining in the adult mouse OE 36–48 h after unilateral right OBX. (A) Unilateral OBX leads to massive apoptosis of olfactory sensory neurons in the ipsilateral OE only, visualized by TUNEL staining (red). The basal lamina (dashed line) separates the OE proper (arrow) from underlying lamina propria (arrowhead). Bar, 200 μ m. (B) The lamina propria contains axon bundles of olfactory sensory neurons, expressing high levels of casp-3 (green). Bar, 200 μ m. (C) After a single BrdU injection, the OE contains only a few scattered BrdU⁺ nuclei next to the basal lamina (green). Bar, 200 μ m. (D) Repeated injections of BrdU over a 24-h period leads to many more BrdU⁺ nuclei, extending toward the surface of the OE. Bar, 200 μ m. (E and F) Combination of BrdU staining (green) and TUNEL labeling (red) in the OE of the latter animal visualized under epifluorescence indicates that colocalization is very rare. Bars, 50 μ m.

et al., 2002; Zhao et al., 2003; Frielingsdorf et al., 2004) and frontal cortex (Gould et al., 2001; Kornack and Rakic, 2001; Koketsu et al., 2003). In the present study, we used BrdU/TUNEL double labeling in established models of injury-induced neuronal cell death to examine the possibility that BrdU labels dying postmitotic neurons in vivo in the adult mouse brain. To obtain the highest level of sensitivity, we used repeated injections or direct infusion of a high dose of BrdU into the brain.

Results

Olfactory bulbectomy (OBX)

We took advantage of the unilateral OBX model (Michel et al., 1994; Holcomb et al., 1995; Bauer et al., 2003) to establish BrdU/TUNEL double-labeling methods. Because neuronal apoptosis occurs only in the olfactory epithelium (OE) ipsilateral to the lesion, this model provides both internal negative and positive controls for the TUNEL reaction (Fig. 1 A). In addition, ongoing proliferation of neuronal progenitors in the basal cell layer of the OE (Schwartz Levey et al., 1991) ensures consistent BrdU incorporation in this tissue. After OBX, animals were killed at the peak of injury-induced apoptosis (36–48 h; Michel et al., 1994; Holcomb et al., 1995), receiving i.p. BrdU injections once 2 h before sacrifice (100 or 300 mg/kg) or 10 times (100 mg/kg) during the 24-h period preceding sacrifice.

Ipsilateral to the lesion, TUNEL⁺ nuclei are located in the middle of the OE (Fig. 1, A, E, and F). They correspond to dying olfactory sensory neurons (Holcomb et al., 1995) whose

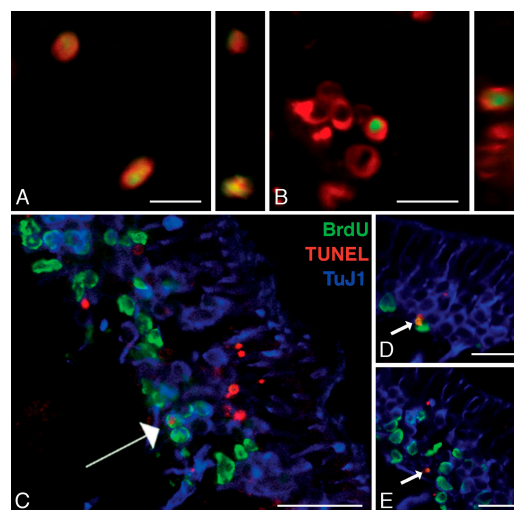


Figure 2. 2 d after unilateral OBX, the OE contains many dying neurons but rare BrdU/TUNEL colocalization, detected only when BrdU is given repeatedly. (A) TUNEL staining (red) colocalizes with BrdU (green) in various patterns with considerable overlap. (B) BrdU staining is shown inside a TUNEL⁺ nucleus. (C) TUNEL staining is shown inside a BrdU⁺ nucleus. (A and B) Vertical images show orthogonal projections along the z axis. (C–E) Colocalization is most often detected in the basal OE, where progenitor cells proliferate and immature neurons (TuJ1 in blue) are generated. Bars: (A) 5 μ m; (B) 10 μ m; (C) 50 μ m; (D and E) 20 μ m.

axons, in the lamina propria beneath the OE, express high levels of activated caspase 3 (casp-3; Fig. 1 B; Cowan et al., 2001). In animals receiving a single BrdU injection, only a few scattered BrdU⁺ nuclei are found next to the basal lamina (Fig. 1 C), where proliferating cells are normally located (Schwartz Levey et al., 1991). Considerably more labeled nuclei, extending toward the surface of the OE, are detected when BrdU is given in repeated injections (Fig. 1 D). In such animals, observation of double labeling under epifluorescent light reveals virtually no colocalization with TUNEL staining (Fig. 1, E and F).

The occurrence of double-labeled cells was carefully investigated using three-dimensional laser confocal microscopy. Despite cases of close apposition between BrdU⁺ and TUNEL⁺ nuclei, no colocalization was detected in animals receiving a single BrdU injection at either dose used (unpublished data). In contrast, in animals subjected to multiple BrdU injections, a few nuclei (up to 25 per section) are simultaneously labeled with BrdU and TUNEL (Fig. 2). In most cases, double-stained nuclei are located in the basal OE next to the lamina propria (Fig. 2, C–E); some also colocalize with TuJ1, a marker of immature neurons (Fig. 2 C).

These results show that BrdU is only marginally detected in dying olfactory sensory neurons after OBX: although extensive TUNEL staining is observed in the OE (>2,500 TUNEL⁺ nuclei per section), <1% of the TUNEL⁺ nuclei are simultaneously labeled with BrdU. Moreover, double-stained nuclei are exclusively found in animals subjected to multiple injections of BrdU and generally reside in the basal OE, where proliferation normally occurs. These observations suggest that BrdU is incorporated in proliferating neuronal precursors before they undergo apoptosis, rather than during the apoptotic

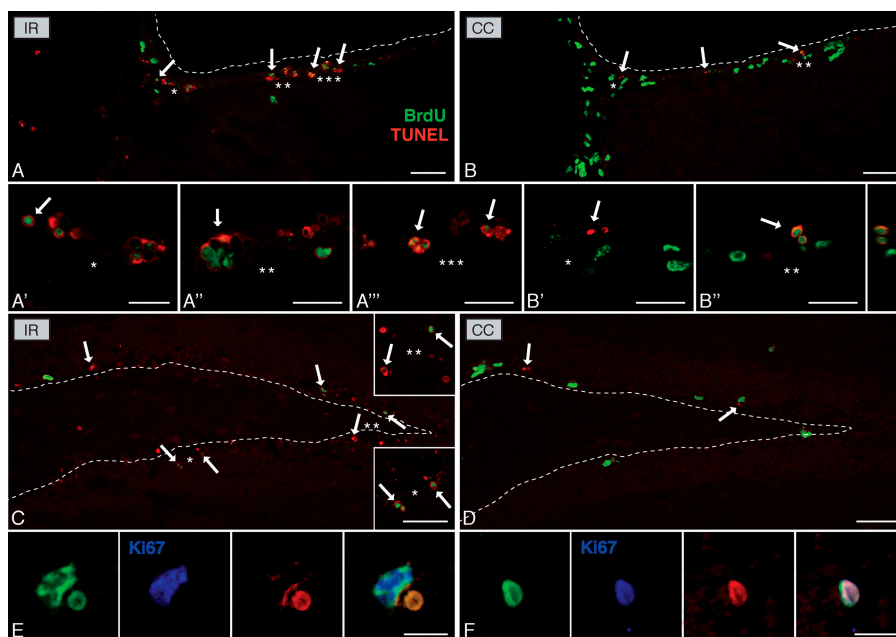


Figure 3. Detection of BrdU and TUNEL staining 8.5 h after brain irradiation shows a large increase in neuronal death but a decrease in BrdU incorporation. Brain irradiation (IR) induces a >44-fold increase in TUNEL⁺ nuclei in the SVZ of the lateral ventricle (A, dotted line) and the SGZ of the hippocampus (C, dotted line). In the control brain (CC), sporadic cell death is detected (arrows). After brain irradiation, BrdU incorporation is reduced more than twofold in both germinal zones (compare B and D with A and C, respectively) and virtually all BrdU⁺ nuclei are simultaneously TUNEL⁺ (A'–A''' and insets in C, corresponding to areas marked by asterisks in A and C, respectively). (B' and B'') BrdU/TUNEL colocalization is also found in the normal adult brain but to a considerably lesser extent and only in the SVZ. (B'') Vertical image shows orthogonal projection along the z axis. (E and F) After brain irradiation, some of the BrdU/TUNEL double-stained nuclei are also positive for Ki67 (blue), indicating cellular proliferation. Bars: (A–D) 50 μ m; (A'–B'') 20 μ m; (C, insets) 30 μ m; (E and F) 10 μ m.

process. This conclusion is supported by extensive colocalization between BrdU and TuJ1 (Fig. 2), as well as by the known susceptibility of these immature neurons to OBX-induced apoptosis (Holcomb et al., 1995).

Brain irradiation

To investigate the possibility that DNA repair is a source of BrdU incorporation not related to cell proliferation, we subjected an adult mouse to brain irradiation, followed by an i.p. injection of 100 mg/kg BrdU at 4, 5, 6, 7, and 8 h after irradiation and killing at 8.5 h (30 min after the last BrdU injection).

In a nonirradiated animal receiving the same regimen of BrdU injection, BrdU labeling is found almost exclusively in areas of ongoing proliferation, i.e., the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus (Fig. 3). Only scarce TUNEL⁺ nuclei are detected in either germinal zone (Fig. 3 and Table I). Interestingly, the noninjured SVZ, but not the SGZ, contains TUNEL⁺ nuclei colabeled with BrdU ($7.8 \pm 3.6\%$ of the total TUNEL⁺ nuclei; Fig. 3 B and Table I). This contrasts with the case of a single BrdU injection where no BrdU/TUNEL colabeling is detected (Cooper-Kuhn and Kuhn, 2002) and could be attributable to the potential toxicity of the drug when administered in repeated injections.

Brain irradiation induces extensive TUNEL labeling, which is also largely restricted to areas of ongoing proliferation (Fig. 3). At the same time, both the SVZ and SGZ display a more than twofold reduction in the number of BrdU⁺ nuclei (Fig. 3 and Table I). Irradiation of the rodent adult brain has been shown to preferentially kill proliferating cells (Peissner et al., 1999). Outside these two germinal zones, only a few nuclei are either BrdU⁺ or TUNEL⁺, and double labeling is very rare (unpublished data). In addition, we never found BrdU staining in postmitotic NeuN⁺ neurons in any brain region examined (unpublished data). In the SVZ or SGZ, >73% of the BrdU⁺ nuclei colocalize with TUNEL staining (Fig. 3 and Table I), reflecting the death of proliferating cells, as well as putative BrdU incorporation attributable to DNA repair/apoptosis. However, a great majority of the dying cells does not incorporate BrdU because only $22.5 \pm 1.6\%$ and $12.8 \pm 2.3\%$ of the TUNEL⁺ nuclei are simultaneously labeled with BrdU in the SVZ and SGZ, respectively (Fig. 3 and Table I). Interestingly, both regions also display some double-stained nuclei that express the proliferation marker Ki67 (Fig. 3, E and F), confirming that a few BrdU/TUNEL⁺ positive cells are proliferating.

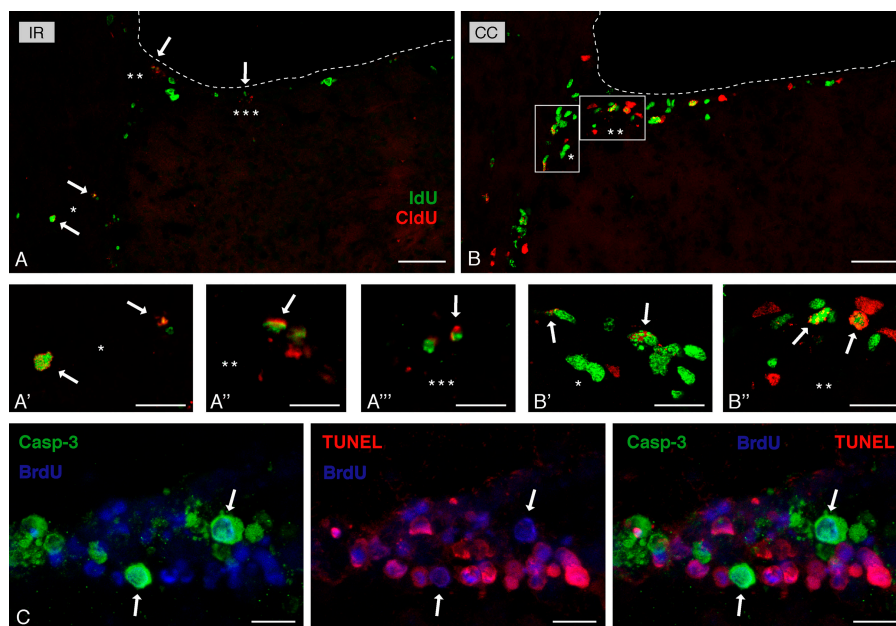
Despite this apparent increase in BrdU/TUNEL colocalization induced by brain irradiation, it is not possible to clearly

Table I. Mean number of positive cells (\pm SEM) for TUNEL, BrdU, or double-labeled BrdU⁺/TUNEL⁺ quantified in the control brain and 8.5 h after brain irradiation in the SVZ and the hippocampal SGZ

Staining	Brain irradiation		Control brain	
	SVZ	SGZ	SVZ	SGZ
BrdU	85.5 ± 2.58	15 ± 2.62	207.33 ± 6.24	33.17 ± 1.3
TUNEL	283 ± 13.63	93.33 ± 9.13	6.33 ± 1.15	0.5 ± 0.34
Double	62.5 ± 2.48	11.67 ± 1.94	0.67 ± 0.33	0
Among TUNEL	$(22.52 \pm 1.64)^a$	$(12.84 \pm 2.33)^a$	$(7.80 \pm 3.63)^a$	$(0)^a$

^aParentheses indicate the ratio of double-labeled cells among the total number of TUNEL⁺ cells.

Figure 4. Detection of IdU and CldU staining 8 h after brain irradiation indicates a reduction in both labels. IdU (green) was injected 1.5 h before irradiation, whereas CldU (red) was injected 6 h after irradiation. (A and B) Brain irradiation (IR) decreases IdU⁺ and CldU⁺ nuclei by 40 and 75%, respectively, in the SVZ of the lateral ventricle (dotted lines) compared with control (CC). Insets in B correspond to high-power magnification pictures showing single IdU⁺ or CldU⁺ cells, as well as double-stained cells in the control SVZ (B' and B'', arrows). After brain irradiation, most of the CldU⁺ nuclei are also labeled with IdU (A'-A'''), corresponding to areas marked by asterisks in A). (C) Brain irradiation induces strong expression of casp-3 (green) in the SVZ, as well as massive TUNEL staining (red). Interestingly, when BrdU is injected repeatedly after brain irradiation, most of the BrdU staining (blue) is found in TUNEL⁺ cells and only in a few caspase 3⁺ cells (arrows). Bars: (A and B) 50 μ m; (A'-B'' and C) 10 μ m.



identify the source of BrdU incorporation because it could occur before cell death during cell proliferation or during DNA repair/apoptosis. In an attempt to differentiate between these two possibilities, we used a double-nucleoside labeling strategy by injecting two thymidine analogues, iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU), before and after brain irradiation, respectively. In this paradigm, IdU⁺ cells are indicative of nucleoside incorporation that relates to cell proliferation before induction of DNA repair, whereas CldU⁺ cells indicate nucleoside incorporation that relates to both cell proliferation and DNA repair/apoptosis. If a significant amount of nucleoside is incorporated during DNA repair, then an increased CldU incorporation (cell proliferation plus DNA repair) should be detected, compared with IdU incorporation (cell proliferation only).

Brain irradiation was performed in one animal that received a single IdU injection 1.5 h before initiating irradiation, followed by a single CldU injection 6 h after irradiation and 2 h before perfusion. By comparing the effects of this procedure with those on a control animal that received the same injection paradigm, we discovered that brain irradiation reduces the number of IdU⁺ and CldU⁺ nuclei by 41 and 75%, respectively (Fig. 4 and Table II), confirming that irradiation kills proliferating cells (IdU⁺) and reduces cell proliferation (CldU⁺). Given that IdU and CldU injections were separated by 8 h, that the bioavailability of IdU and CldU may be similar to BrdU (at least 2 h; Takahashi et al., 1992), and that the cell cycle for SVZ cells has been estimated to last ~ 12.7 h with an S phase of ~ 4.2 h (Morshead and van der Kooy, 1992), IdU/CldU double-stained cells represent cells that are still in the S phase (labeled during the same cell cycle), as well as CldU-incorporating, proliferating daughter cells of a progenitor that had incorporated IdU in the previous cell cycle. Brain irradiation reduces the number of IdU/CldU double-stained nuclei by 52%, which is

slightly more than the reduction of IdU⁺ cells alone (41%; indicating death of proliferating cells), suggesting that irradiation also prevents progenitors from reentering the cell cycle. Interestingly, although $\sim 50\%$ of the CldU⁺ nuclei are also IdU⁺ in the control animal, virtually all (91%) of the CldU⁺ nuclei colocalize with IdU after irradiation (Table II). This indicates that most of the cells labeled with CldU after irradiation were in the S phase of the cell cycle before irradiation, which could represent cells that either terminate their S phase or exit the S phase and incorporate CldU during DNA repair. The latter should therefore lead to an increased CldU staining in IdU⁺ cells compared with the control animal. However, the proportion of CldU⁺ nuclei among IdU⁺ cells is slightly reduced after irradiation (28%) versus control (35%), indicating that DNA repair/apoptosis does not trigger an increased CldU incorporation in cells that were proliferating in healthy condition. This lack of increased

Table II. Mean number of positive cells (\pm SEM) for IdU, CldU, TUNEL, or double-labeled (IdU⁺/CldU⁺, IdU⁺/TUNEL⁺, or CldU⁺/TUNEL⁺) cells quantified in the control brain and 8 h after brain irradiation in the SVZ

Staining	Brain irradiation	Control brain
IdU	95.5 \pm 6.24	161.75 \pm 1.75
CldU	30 \pm 3.34	120 \pm 8.29
TUNEL	416.63 \pm 9.3	5.13 \pm 0.71
IdU/CldU	27 \pm 2.35	56.5 \pm 7.24
Among IdU	(28.56 \pm 2.97) ^a	(34.85 \pm 4.17) ^a
Among CldU	(91 \pm 3) ^a	(46.82 \pm 4) ^a
IdU/TUNEL	82.25 \pm 5.28	0.5 \pm 0.22
Among IdU	(88.77 \pm 2.77) ^a	(0.46 \pm 0.15) ^a
Among TUNEL	(19.23 \pm 1.5) ^a	(14.44 \pm 8.01) ^a
CldU/TUNEL	22.5 \pm 1.94	0
Among CldU	(76.66 \pm 4.81) ^a	(0) ^a
Among TUNEL	(5.55 \pm 0.34) ^a	(0) ^a

^aParentheses indicate the ratio of double-labeled cells among the total number of IdU⁺, CldU⁺, or TUNEL⁺ cells, as indicated in the left column.

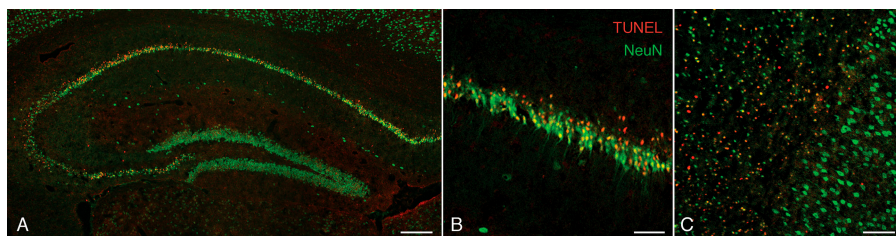


Figure 5. KA-induced seizure in adult FVB/N mice leads to massive neuronal cell death. Postmitotic neurons (NeuN, green) are extremely TUNEL⁺ (red) in the CA region of the hippocampus (A and B) and in the somatosensory cortex (C) by 3 d after lesion. Bars: (A) 200 μ m; (B) 50 μ m; (C) 100 μ m.

CldU incorporation occurs despite extensive cell death of the IdU⁺ cells because $88.8 \pm 2.8\%$ are also TUNEL⁺ after irradiation, which is in fact slightly more than what is seen for CldU⁺ cells ($76.7 \pm 4.8\%$ are also TUNEL⁺). These results indicate that CldU does not label a greater proportion of dying cells even when this nucleoside is administered during DNA repair/apoptosis. In fact, only $5.6 \pm 0.3\%$ of the TUNEL⁺ cells are also CldU⁺, whereas this value is much greater for IdU⁺ cells ($19.2 \pm 1.5\%$) and similar to what is found with the repeated BrdU injections (22.5%), suggesting that in this experiment most of the BrdU incorporation is related to cell proliferation.

Altogether, our data indicate that the massive induction of DNA repair *in vivo* does not lead to a detectable increased incorporation of BrdU or related nucleoside. It could be argued, however, that DNA repair is completed quickly after brain irradiation and that we may miss this event by injecting BrdU or CldU 4–6 h after irradiation. We checked for this possibility in an animal that received an injection of CldU immediately after brain irradiation, 1 h before perfusion. No difference in cell death or CldU labeling was detected when compared with control (unpublished data).

Because TUNEL staining mostly labels cells in late stages of apoptosis, we also investigated the occurrence of BrdU incorporation in dying cells at an earlier stage of the apoptotic process by staining for casp-3. Although the control SVZ and SGZ contain only a few casp-3⁺ cells, brain irradiation induces strong casp-3 staining in both areas (Fig. 4). Interestingly, however, very few of the BrdU⁺ cells in the SVZ are also casp-3⁺ ($18.8 \pm 2.1\%$) in the repeated injection experiment, which strongly contrasts with our observation for TUNEL staining (>73%; Fig. 4 and Table I). This difference might relate to variable cellular sensitivity to irradiation depending on cell cycle kinetics (Shinomiya, 2001), and one hypothesis is that most casp-3⁺ cells were not proliferating and did not incorporate BrdU. This observation may also reflect the radiosensitizing effect of BrdU (McGinn et al., 1996), which suggests a general toxicity of BrdU for cells undergoing DNA repair. BrdU could lead to an induction or acceleration of the cell death process, so that BrdU-containing cells would quickly resume apoptosis, fragment their DNA, and be cleared. Interestingly, mature neurons subjected to ischemic preconditioning can survive *in vivo* even after expressing high levels of casp-3 (Tanaka et al., 2004). Our observation therefore makes it very unlikely that BrdU would label a casp-3⁺ neuron, which could survive and lead to false positive BrdU staining. Whether a TUNEL⁺ cell can survive *in vivo* is currently unknown.

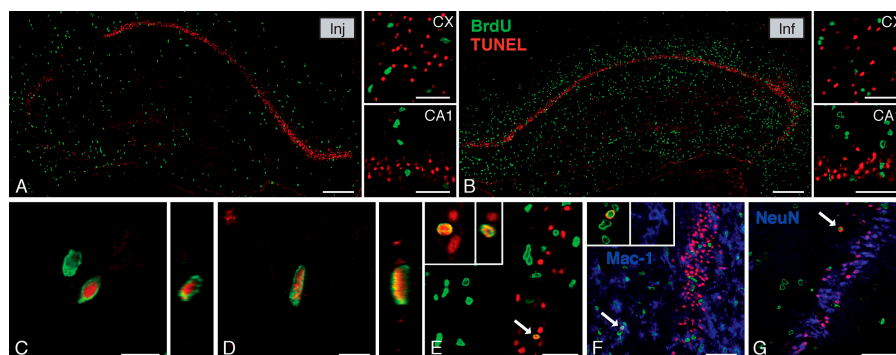
Kainic acid (KA)-induced seizure

After OBX and brain irradiation, cell death is induced in compartments where progenitor cells proliferate, preventing a clear interpretation of the results. We therefore used the excitotoxic model of KA-induced seizure, which triggers activation of DNA repair mechanisms and death of postmitotic hippocampal pyramidal neurons (Morrison et al., 1996; Djebaili et al., 2001; Tan et al., 2002). BrdU was either given as a single i.p. injection (100 mg/kg) 2 h before killing, at 1 or 3 d after KA injection, or directly infused into the lateral ventricle (Pencea et al., 2001; Zhao et al., 2003) for a 24-h period before killing, 4 d after KA injection. The infusion of BrdU was used to maximize sensitivity because it leads to a much higher uptake into brain cells than i.p. injection (Pencea et al., 2001).

As previously documented for FVB/N mice (Faherty et al., 1999), cell death is not detected in the cornu ammonis (CA) region of the hippocampus 1 d after KA-induced seizure. Only sparse TUNEL staining is visible in the amygdala, piriform cortex, and somatosensory cortex (unpublished data). However, cell death dramatically increases 3–4 d after KA injection (Figs. 5 and 6). At this time, massive TUNEL staining, extensively colocalizing with NeuN labeling, is found throughout the CA region of the hippocampus and the somatosensory cortex (layers I–III; Fig. 5), as well as in the piriform and rhinal cortices, striatum, and amygdala (not depicted). In this context of neurodegeneration, sparse but numerous BrdU⁺ nuclei are detected throughout the brain after a single BrdU injection (Fig. 6 A). In contrast, infusion of BrdU into the lateral ventricle dramatically increases the density and number of BrdU⁺ nuclei in areas surrounding the ventricles (Fig. 6 B).

In spite of numerous examples of close apposition between BrdU⁺ and TUNEL⁺ nuclei, colocalization is not detected in any of the damaged brain areas in animals receiving a single BrdU injection either 1 or 3 d after KA injection (Fig. 6 A and Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200505072/DC1>). In the BrdU-infused animal, a general absence of colocalization is similarly seen (Fig. 6 B), despite a considerably greater number of BrdU⁺ nuclei and an apparent progression of cell death in the hippocampus (Fig. 6, A and B; and Fig. S2). Nevertheless, rare double-stained nuclei are detectable (<5 colocalizations out of >4,000 TUNEL⁺ nuclei per section) exclusively in regions near the lateral ventricle such as the SVZ, where some of them feature the elongated/bipolar shape typical of migrating neurons (Fig. 6, C and D). In the CA3 area, a total of three BrdU⁺/TUNEL⁺ nuclei are detectable in six sections (Fig. 6 E), whereas no colocalization is seen in CA1. Most of the double-stained nuclei in the hippocampus are present in the stratum radiatum and colocalize with

Figure 6. BrdU and TUNEL staining 3–4 d after KA injection shows a major increase in neuronal death but rare double labeling. (A and B) BrdU was either injected i.p. (100 mg/kg) 2 h before killing (Inj) or directly infused in the lateral ventricle for 24 h (Inf). Massive cell death is detected in both the hippocampus (A, B, and CA1) and the somatosensory cortex (CX). (A) However, BrdU/TUNEL colocalization is absent in animals receiving a single BrdU injection. (B) Similarly, after infusion of BrdU, which leads to a much higher uptake of the marker into brain cells, the majority of dying cells is not positive for BrdU. Rare colocalization is nonetheless detectable in this case, specifically in areas close to the lateral ventricle, such as the SVZ (C) or the corpus callosum (D). A total of three BrdU⁺/TUNEL⁺ nuclei are detected in the CA3 area in six sections containing >24,000 TUNEL⁺ nuclei (E, arrow and insets). In the hippocampal formation, most of the double-stained nuclei are found in the stratum radiatum and colocalize with Mac-1, a marker of microglia (blue in F, arrow and insets) but not with NeuN (blue in G, arrow). Vertical images represent orthogonal projections along the z axis. Insets in E and F are 18 and 34 μm wide, respectively. BrdU is in green, and TUNEL is in red. Bars: (A and B) 200 μm ; (C and D) 10 μm ; (E) 30 μm ; (CA1, CX, F, and G) 50 μm .



the microglia marker Mac-1 (Fig. 6 F). No colocalization between BrdU, TUNEL, and NeuN is detected (Fig. 6 G). BrdU/TUNEL colocalization is absent in the somatosensory cortex, rhinal cortex, and amygdala (unpublished data).

To assess possible activation of DNA repair or cell cycle mechanisms in injured neurons, we looked at the expression of p53 and cyclin D1, respectively. In the CA1/CA3 area and the somatosensory cortex, some postmitotic NeuN⁺ neurons display high expression of nuclear p53 protein 3 d after KA injection (but not at 1 d) but are never positive for BrdU (Fig. 7, A–D). Surprisingly, we find strong nuclear cyclin D1 immunoreactivity in specific populations of postmitotic neurons in intact control mice (FVB/N and C57BL/6), including CA1 pyramidal neurons, where cyclin D1 immunoreactivity abruptly stops at the junction with CA2/CA3 (Fig. 7, E and F), as well as in neurons of the piriform cortex (Fig. 7 G) and amygdala (not depicted). As soon as 1 d after KA injection, cyclin D1 immunoreactivity disappears from these structures, probably because of the progression of cell death, but increases at 3–4 d in cortical reactive astrocytes (Fig. 7 H).

Together, these data demonstrate that BrdU is not significantly incorporated in vivo in dying postmitotic neurons after an excitotoxic lesion. Although some of the dying cells appear simultaneously positive for BrdU (<1%), their characteristics suggest that BrdU was incorporated during proliferation before cell death rather than during apoptosis/DNA repair.

Discussion

Our examination of three different models of injury-induced neuronal apoptosis in vivo indicates that BrdU incorporation is not detected in dying postmitotic neurons. This suggests that the amount of BrdU that would be incorporated during normal, ongoing neuronal cell death in vivo is below the detection limit of immunohistochemistry.

BrdU incorporation in terminally differentiated neurons undergoing cell death could occur during DNA repair or reentry into the cell cycle (Nowakowski and Hayes, 2000; Rakic, 2002a,b). Given the recently demonstrated relationship be-

tween these two mechanisms (Kruman et al., 2004), experimentally identifying the source of BrdU incorporation during the cell death process is difficult. In a mouse model of KA-induced excitotoxicity, we find that BrdU is not detected in dying terminally differentiated neurons (i.e., TUNEL⁺) or in vulnerable neurons likely undergoing DNA repair (i.e., expressing nuclear p53 protein). The absence of cyclin D1 expression, a regulator of the cell cycle involved in the progression from G1 to S phase, contrasts with what has been described in the adult rat brain (Ino and Chiba, 2001) but could be attributable to the use of different species or antibodies. In addition, we may have missed an early cyclin D1 induction because it has been detected in CA3 neurons 12 h after KA injection in CD1 mice (Park et al., 2000). Nevertheless, our results are consistent with a recently published study using the same model, which also failed to detect BrdU incorporation in TUNEL⁺ neurons after a single BrdU injection (Kuan et al., 2004). Most important, we further extend these data by showing that even the direct cerebral infusion of a high dose of BrdU does not result in the labeling of dying postmitotic neurons, which indicates that increasing the dose of BrdU does not contribute to nonspecific staining. Infusion of BrdU was recently used to detect low levels of neurogenesis in the substantia nigra (Zhao et al., 2003; Frielingsdorf et al., 2004), whereas neurogenesis was not detected using an i.p. injection (Lie et al., 2002).

Could BrdU incorporation in injured terminally differentiated neurons in vitro be an artifact?

Recent studies (Greene et al., 2004) indicate that BrdU incorporation in postmitotic neurons is primarily found in cultured embryonic cortical neurons (Copani et al., 1999; Wu et al., 2000; McPhie et al., 2003; Kruman et al., 2004) or early postnatal cerebellar granule cells (Verdaguer et al., 2002) that are injured by various means. In contrast, BrdU incorporation in vulnerable or dying neurons in vivo has rarely been directly demonstrated after injury or during physiological programmed cell death, although activation of cell cycle-related proteins is commonly observed (Greene et al., 2004). Only indirect obser-

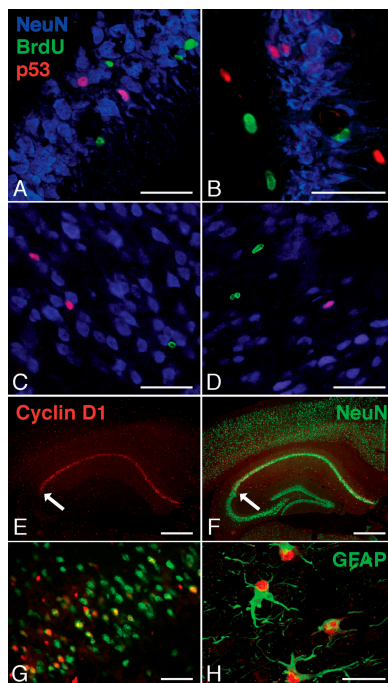


Figure 7. Markers of DNA repair (p53 protein) and cell cycle (cyclin D1) do not label BrdU⁺ cells. After KA injection, a few NeuN⁺ postmitotic neurons (blue) express a high level of nuclear p53 protein (A–D, red) but are never positive for BrdU (green), either in the CA1/CA3 areas (A and B) or the cortex (C and D). Surprisingly, the intact adult mouse brain (E–G) contains populations of postmitotic neurons (NeuN⁺, green) showing a high level of nuclear cyclin D1 (E–H, red) immunoreactivity, including pyramidal neurons of the CA1 area (E and F), where cyclin D1 immunoreactivity abruptly stops at the junction with CA2/CA3 (E and F, arrows), and neurons of the piriform cortex (G). After KA injection, cyclin D1 is highly expressed in hypertrophic reactive glial fibrillary acidic protein (GFAP⁺) astrocytes (green) in the cortex (H). Bars: (A–D and G) 50 μ m; (E and F) 500 μ m; (H) 30 μ m.

variations such as a spatial correlation between proliferating and dying neurons or the pycnotic aspect of a BrdU⁺ nucleus were suggestive of BrdU incorporation in dying or committed-to-death postmitotic neurons (Lee et al., 1992; Herrup and Busser, 1995; Klein et al., 2002; El-Khodori et al., 2003). Menu dit Huart et al. (2004) indicated BrdU/TUNEL double staining by “data not shown.” Moreover, such *in vivo* observations were exclusively made during embryonic development or early postnatal life (except by Klein et al., 2002). In the adult brain, only a few studies have directly evaluated possible BrdU incorporation in dying postmitotic neurons after injury, including adrenalectomy, traumatic brain injury, hypoxia, ischemia, and KA-induced excitotoxicity (Katchanov et al., 2001; Tonchev et al., 2003a,b; Kuan et al., 2004). Interestingly, although each of these insults produced virtually no BrdU labeling in dying neurons when performed separately, the combination of ischemia and hypoxia was recently reported to induce terminally differentiated neurons to reenter the cell cycle and incorporate BrdU (Kuan et al., 2004). This suggests that the DNA damage response induced by a single insult, such as ischemia or excitotoxicity, may not be sufficient to trigger cell cycle reentry of terminally differentiated neurons, whereas a combination of insults (ischemia plus hypoxia) could synergistically activate

specific components of the DNA damage response that trigger aberrant cell cycle reactivation in postmitotic neurons *in vivo*. Additionally, experimental models that induce neuronal cell death may not be efficient in inducing cell cycle reactivation of postmitotic neurons, which could also rely on different susceptibility to DNA damage among various neuronal populations (Pieper et al., 2000). Nevertheless, in the normal adult brain, or when dealing with classical injury models of acute neuronal cell death, BrdU appears to be a specific and reliable marker for studying neurogenesis, even when a high dose of BrdU is directly infused into the brain.

Reactivation of the cell cycle and BrdU incorporation in neurodegenerative disease

In neurodegenerative disorders such as AD or amyotrophic lateral sclerosis, at-risk neurons express cell cycle-related proteins such as proliferating cell nuclear antigen, cyclin D1, Cdk4, pRb, cyclin B, or cdc2 (Greene et al., 2004). Surprisingly, however, many of these proteins are cytoplasmically localized (Bowser and Smith, 2002). Furthermore, proliferating cell nuclear antigen, which is considered as a marker for cycling cells, is also involved in DNA repair (Uberti et al., 2003) and can be expressed in postmitotic neurons (Ino and Chiba, 2000). We find that cyclin D1 is specifically expressed at relatively high levels in some neuronal populations of the control mouse brain, where it is localized in the nucleus, as is seen in cycling cells. This result was unexpected because these neuronal populations are clearly noncycling and postmitotic, and it suggests that cyclin D1 might have a role distinct from its classic regulation of the progression into the cell cycle. That is, the up-regulation of cell cycle-related proteins seen in neurodegenerative disorders is not directly conclusive of cell cycle reactivation.

Recently, Yang et al. (2001) used FISH to show that some vulnerable neurons in human brains with AD are aneuploid, which was interpreted as evidence of DNA replication preceding neuronal cell death. However, the FISH technique gives an indication of the amount of nuclear DNA, haploid to tetraploid depending on the number of hybridization spots (Yang et al., 2001), and does not indicate recent or abnormal replication or proliferation. Other mechanisms that could explain the presence of aneuploid cells in brains with AD, such as cell fusion (Alvarez-Dolado et al., 2003), should be considered. Most important, recent studies in the normal mouse have shown that embryonic and postnatal neural progenitor cells, as well as 5–7% of the neurons in the adult brain, are aneuploid (Kaushal et al., 2003; McConnell et al., 2004). Constitutive aneuploidy has also been recently reported in the normal adult human brain (Rehen et al., 2005). The study by Yang et al. (2001), however, did not find such abnormalities in age-matched control brains, suggesting that aneuploidy might be increased in AD. According to this hypothesis, AD would be characterized by the failed elimination of aneuploid neurons, which could even accumulate slowly with age during adult neurogenesis (Eriksson et al., 1998; Jin et al., 2004) and lead to functional disturbances of the brain. In this context, it is interesting to note that mice lacking ataxia-

telangiectasia, a protein involved in the control of DNA repair, cell cycle, and apoptosis (Shiloh, 2003), fail to eliminate aneuploid neurons (McConnell et al., 2004) and display age-dependent neurodegeneration in the substantia nigra and striatum (Eilam et al., 2003). Moreover, recent analyses of a mouse model of AD characterized by neuronal loss (APP23 [amyloid precursor protein transgenic mice]; Calhoun et al., 1998) have shown that cortical neurons do not incorporate BrdU or express markers of the cell cycle, whereas astrocytes do (Bondolfi et al., 2002; Gartner et al., 2003).

Injury-induced neuronal death could occur through pathways that are distinct from naturally occurring cell death, especially during slow, progressive neurodegenerative disorders such as AD. Interestingly, a recent study of the normal development of the rabbit cerebellum suggests that apoptosis of granule cells is controlled by two distinct mechanisms, one involving the activation of cell cycle proteins in premigratory neurons and the other being the more classic caspase-dependent pathway in postmigratory neurons, without reactivation of the cell cycle (Lossi et al., 2004). This observation could explain why many *in vitro* studies, which use embryonic or neonatal neurons, as well as *in vivo* studies using perinatal animals, report BrdU incorporation in dying neurons. Within the adult brain, neurons may behave differently, and despite an up-regulation of cell cycle-related proteins, our results as well as those of others (Katchanov et al., 2001; Tonchev et al., 2003a,b) demonstrate that dying postmitotic neurons rarely progress through S phase or incorporate BrdU.

Materials and methods

Animals

2- to 4-mo-old C57BL/6 mice (The Jackson Laboratory) were subjected to unilateral OBX (Bauer et al., 2003) or brain irradiation with a Cesium¹³⁷ source calibrated to deliver 20 grays in 25 min (Mark I-68A; J.L. Shepherd & Assoc.). FVB/N mice received a subcutaneous injection of 30 mg/kg KA (Sigma-Aldrich) dissolved in saline and were carefully monitored for seizure activity. Mice undergoing status epilepticus for at least 4 h were analyzed. Seizures were stopped by an *i.p.* injection of 10 mg/kg diazepam (Steris Laboratories) as needed.

Treatments with thymidine analogues

BrdU and CldU (Sigma-Aldrich) were dissolved in 16.67 mg/ml saline and IdU (Sigma-Aldrich) in 10 mg/ml PBS/NaOH, pH ~8.0. Solutions were sterile filtered and injected *i.p.* or delivered by intracerebroventricular infusion with a minipump (1 μ l/h; model 1003D; Alzet) attached to a Brain infusion kit II (Alzet), with the cannula implanted in the right lateral ventricle (lateral, 0.8 mm; anterior, -0.1 mm to the bregma; depth, 3 mm from skull surface). The pump was left in place for 24 h, delivering a total of 0.4 mg BrdU.

Double staining for BrdU and TUNEL

For BrdU/TUNEL double staining, TUNEL labeling was performed first, as described previously (Bauer et al., 2003), and revealed with streptavidin Alexa 568 (Invitrogen). Sections were fixed for 30 min at RT in 4% PFA in 0.1 M phosphate buffer, pH 7.4, and rinsed with PBS. BrdU staining was done using the HCl method as described previously (Bauer et al., 2005) and revealed with Alexa 488 goat anti-rat (Invitrogen). Additional staining was performed either before the TUNEL reaction or after BrdU staining and revealed with Alexa 633-conjugated secondary antibodies.

Double staining for IdU and CldU

Anti-BrdU antibodies cross-reacting with CldU (Immunologicals.com) or IdU (BD Biosciences) were incubated overnight at RT in PBS containing 10% normal goat serum, 5% BSA, and 0.1% Triton X-100 [CldU first, followed by IdU; Maslov et al., 2004]. Possible cross-reactivity was removed by wash-

ing 6×10 min with a high-stringency solution (40 mM Tris-HCl, 50 mM NaCl, and Tween 1%, pH 8.0). Specificity was checked using brain sections from animals injected with IdU or CldU and stained for CldU or IdU, respectively, which gave no detectable staining. This control procedure was repeated systematically when we performed the double staining. We also compared the sensitivity of IdU, CldU, and BrdU incorporation/detection by quantifying cells labeled in the SVZ of animals injected with 100 mg/kg of either thymidine analogue 2 h before perfusion. The number of IdU- or CldU-labeled cells was within 5–7% of the number of BrdU⁺ cells, whereas only a 1% difference was seen between IdU⁺ and CldU⁺ cells.

Triple-staining immunohistochemistry

Primary antibodies used include mouse anti-TuJ1 (Covance), mouse anti-Ki67 (BD Biosciences), mouse anti-NeuN (Chemicon), rat anti-Mac-1 (Biosource International), rat anti-glial fibrillary acidic protein (Zymed Laboratories), rabbit anti-p53 (Novocastra), rabbit monoclonal anti-cyclin D1 (a gift from C. Pagtakhan, Lab Vision, Fremont, CA), and rabbit anti-casp-3 (Cell Signaling Technology). Antibodies were incubated overnight at 4°C or RT in PBS containing 0.1% Triton X-100 and 10% normal goat serum. Species-specific secondary antibodies conjugated to Alexa 488, 568, or 633 were purchased from Invitrogen. Nonspecific staining was systematically checked by omission of the primary antibody. For p53 staining, sections were first incubated for 10 min in boiling 0.01 M citrate buffer, pH 6.0. For cyclin D1 staining, additional control consisted of preincubating the antibody with the synthetic peptide antigen (Lab Vision) for 1 h at RT, resulting in the disappearance of the staining in a concentration-dependent manner. In addition, a mouse monoclonal anti-cyclin D1 (Santa Cruz Biotechnology, Inc.) gave similar staining in the hippocampus, although much weaker and with less contrast.

Confocal microscopy and quantification

Double or triple staining was analyzed in three dimensions with an LSM 410/Axiovert 135 system (Carl Zeiss Microimaging, Inc.) equipped with three laser lines (488, 568, and 633 nm). Confocal photomicrographs were acquired with the LSM software version 3.993 (Carl Zeiss Microimaging, Inc.) at a size of 512×512 or 768×576 pixels by averaging 8–16 scans. Each channel was acquired separately using specific laser lines to avoid bleed through of the fluorochromes. Objectives used had a magnification of $10\times$ (NA 0.3), $20\times$ (NA 0.75), or $63\times$ (oil immersion, NA 1.25). In Figs. 5 and 6, confocal photomicrographs were acquired at $10\times$, and the hippocampal formation was reconstructed by assembling two to three pictures in Photoshop version 7.0.1 (Adobe Systems, Inc.). Nonconfocal photomicrographs were acquired with a Spot RT Slider charge-coupled device camera controlled by the Spot software version 3.4 (Diagnostic Instruments) and attached to a microscope (Diaphot 300; Nikon). For all stainings, Photoshop version 7.0.1 was used to adjust contrast and brightness so that individual pictures on the same composite figure appeared similar. Nuclei showing clear colocalizations between thymidine analogues and TUNEL staining were quantified and recorded at the confocal microscope. Quantification was also performed on the microscope using a $63\times$ objective (oil immersion, NA 1.4). At least six sections per animal per condition were analyzed for qualitative purposes, and at least two sections were used for quantification.

Online supplemental material

Figs. S1 and S2 show the extent of cell death and BrdU incorporation in the cortex and hippocampus, respectively, after KA-induced seizures. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200505072/DC1>.

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